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Inhibition of Growth of a *Graphium* sp. on Gaseous *n*-Alkanes by Gaseous *n*-Alkynes and *n*-Alkenes

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The growth of a filamentous fungus, a *Graphium* sp., on *n*-alkanes (C_2 to C_4) was inhibited by low concentrations of acetylene, propyne, 1-butyne, ethylene, and propylene. Acetylene and other unsaturated hydrocarbons had no effect on the growth of the *Graphium* sp. on potato dextrose broth, ethanol, or acetate. Our results suggest that *n*-alkynes and *n*-alkenes are selective inhibitors of a nonspecific monooxygenase enzyme responsible for the initial oxidation of *n*-alkanes.

Graphium sp. strain ATCC 58400 is an example of the limited number of fungal species that grow on gaseous *n*-alkanes (1, 2, 10, 11, 15, 18). This organism was first isolated from an enrichment culture which used natural gas as a carbon and energy source (18). Originally it was suspected that this organism grew on methane (18), although subsequent studies demonstrated that its growth was being supported by the low levels of ethane found in domestic natural gas supplies (15, 16). It has also been demonstrated that this *Graphium* sp. and other fungal species also grow on other gaseous saturated hydrocarbons, including propane and *n*-butane. In contrast to these earlier studies, the aim of the present study was to examine the interaction between the *Graphium* sp. and gaseous unsaturated hydrocarbons. Our results demonstrate that gaseous unsaturated hydrocarbons do not support the growth of the *Graphium* sp. but that they are effective inhibitors of the growth of this organism on gaseous *n*-alkanes.

In all experiments the *Graphium* sp. was grown for 5 to 7 days under axenic conditions in shake flasks (500 ml). The growth medium (100 ml) was either potato dextrose broth (PDB) (24 g/liter) or a mineral salts medium containing (per liter) $(NH_4)_2SO_4$ (1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.1 g), and $FeCl_3 \cdot 6H_2O$ (0.58 mg) added as a solution in 50 mM Na EDTA, pH 7, and 1 ml of a trace elements solution (4). The medium was adjusted to an initial pH of 7 by the addition of 1 ml of an aqueous sterile solution of K_2HPO_4 (10% [wt/vol]). The flasks were inoculated with conidia (between 10^5 and 10^6/100 ml) obtained from mycelia grown for 6 to 8 days at 25°C on potato dextrose agar plates under constant illumination. Following inoculation, the flasks were stoppered with butyl rubber stoppers, and sterile gaseous hydrocarbon substrates (10% gas phase [vol/vol]) were added as an overpressure by using plastic syringes fitted with sterile needles and Acrodisc filters (Gelman Sciences, Ann Arbor, Mich.). The flasks were incubated at 24°C in an orbital shaker (125 rpm). Yields of mycelia were determined from dry weight measurements which were made following vacuum filtration of the culture medium through paper filters (VWR Scientific, Los Angeles, Calif.) which had been previously dried and weighed. After filtration, the filter papers and mycelia were dried (65°C for 24 h) and reweighed. The data presented are representative results of experiments which were typically repeated three times with different conidial preparations. Although the same trends were consistently observed in each repeated experiment, various factors, including variations in the levels of inoculum, percent germination, and incubation period, prevent direct comparisons of yields between experiments.

Initial attempts to grow the *Graphium* sp. in a minimal mineral salts medium with either ethylene or acetylene (10% gas phase [vol/vol]) as potential energy and carbon sources were unsuccessful. However, in several preliminary experiments we noted that low concentrations of acetylene strongly inhibited the growth of the *Graphium* sp. on ethane. Subsequently, we examined the effect of acetylene concentration on the growth of the *Graphium* sp. on either ethane or PDB. Acetylene at concentrations up to 2% gas phase (vol/vol) had no effect on the yield of mycelia obtained from growth of the *Graphium* sp. on PDB, while concentrations of acetylene as low as 0.5% gas phase (vol/vol) inhibited the growth of the *Graphium* sp. on ethane by 95% or more (Table 1). The yield of mycelia grown on ethane (10% gas phase [vol/vol]) for 7 days in the absence of acetylene (ca. 25 mg [dry weight]/100 ml) is comparable to the yields obtained with other ethane-utilizing fungal isolates (11). Previous studies with ethane-utilizing fungal isolates (11) have indicated that the pathway of ethane oxidation most likely involves the sequential production and consumption of ethanol, acetaldehyde, and acetate. The addition of acetylene (0.5% gas phase [vol/vol]) had no effect on the yield of mycelia obtained when the *Graphium* sp. was grown on either ethanol or acetate (20 mM) (Table 2). No growth was observed in the presence of acetaldehyde (20 mM), either with or without acetylene. This effect probably reflects a toxicity of this compound at the concentrations used. Acetylene (0.5% gas phase [vol/vol]) also prevented the growth of the *Graphium* sp. on other gaseous *n*-alkanes such as propane and *n*-butane (Table 3). We also observed that other *n*-alkynes (propyne and 1-butyne) and two *n*-alkenes (ethylene and propylene) were also effective inhibitors of the growth of the *Graphium* sp. on ethane (Table 4). In contrast, the same concentrations of these compounds had no effect on the yield of mycelia obtained with growth on either PDB (Table 4), ethanol, or acetate (data not shown).

The data presented in this report provide the first evidence of the effects of acetylene on a fungal catalyzed process, and our results establish the following four points. First, the *Graphium* sp. does not appear to be capable of growth on unsaturated gaseous hydrocarbons, such as acetylene (Tables 2 and 3) and gaseous *n*-alkenes (Table 4). Second, acetylene is an effective growth inhibitor only when the *Graphium* sp. is

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TABLE 1. Effect of acetylene on growth of the *Graphium* sp. on ethane and PDB

Acetylene concn (% gas phase [vol/vol])	Yield of mycelia on growth substrate (mg [dry wt]/100 ml)	
	MS[a] + ethane[b]	PDB
0	23.7	164.9
0.1	8.4	170.6
0.25	2.2	169.2
0.5	1.8	175.1
1.0	1.0	184.5
2.0	0	177.5

[a] MS, mineral salts medium.
[b] Ethane was present at 10% gas phase (vol/vol).

grown on gaseous *n*-alkanes (Tables 1 and 2). Third, the inhibitory action of acetylene appears to be directed at the initial step in ethane oxidation since acetylene does not affect mycelial growth on the likely initial (ethanol) or late (acetate) products of ethane oxidation (Table 2). Fourth, low concentrations of other unsaturated hydrocarbons, including ethylene, also exhibit an inhibitory effect similar to that of acetylene on the growth of the *Graphium* sp. on ethane. Likewise, none of these compounds affect mycelial growth on PDB (Table 4).

There are numerous effects of acetylene on metalloenzymes and the growth of bacteria (7). For example, acetylene is an alternative substrate for nitrogenase (3) and a reversible inhibitor of hydrogenase (6) and nitrous oxide reductase (17). In contrast, ethylene and other *n*-alkenes are essentially unreactive towards these enzymes. Acetylene and other *n*-alkynes are also potent mechanism-based inactivators of several microbial monooxygenases, including those enzymes responsible for growth substrate oxidation in bacteria which oxidize ammonia (9), methane (13), and propylene (4). Once again, *n*-alkenes are much less inhibitory than acetylene towards these microbial processes. In the case of propylene oxidizers, gaseous *n*-alkenes are often growth-supporting substrates, while the same compounds are often non-growth-supporting substrates (cometabolites) for bacteria which oxidize ammonia (8) and methane (5). In contrast to the general lack of inhibitory effects of gaseous *n*-alkenes on microbial processes, our present results demonstrate the less common situation in which both acetylene (and related *n*-alkynes) and ethylene (and related *n*-alkenes) are inhibitors of the same process at similar low concentrations. To date, the *n*-alkane-oxidizing enzyme system of the *Graphium* sp. has not been extensively studied. However, studies with fungi which utilize gaseous hydrocarbons (1) and longer-chain *n*-alkanes (14) indicate that fungal hydrocarbon oxidations are typically initiated by cytochrome P-450-type enzymes. The presence of an *n*-alkane-oxidizing cytochrome P-450 system in *n*-alkane-grown mycelia of the *Graphium* sp. would be compatible with our results because acetylene, ethylene, and their close homologs are all known as mechanism-based inactivators of cytochrome P-450 enzymes (12).

TABLE 2. Effect of acetylene on growth of the *Graphium* sp. on ethane and potential ethane oxidation products

Growth substrate	Yield of mycelia (mg [dry wt]/100 ml)	
	Without acetylene	With acetylene[a]
MS[b]	2.2	1.5
MS + ethane[c]	30.9	0.4
MS + ethanol (20 mM)	38.1	37.4
MS + acetaldehyde (20 mM)	0.9	0.2
MS + sodium acetate (20 mM)	25.5	26.6

[a] Acetylene was present at 0.5% gas phase (vol/vol).
[b] MS, mineral salts medium.
[c] Ethane was present at 10% gas phase (vol/vol).

TABLE 3. Effect of acetylene on growth of the *Graphium* sp. on *n*-alkanes

Growth substrate	Yield of mycelia (mg [dry wt]/100 ml)	
	Without acetylene	With acetylene[a]
MS[b]	2.8	3.2
MS + ethane[c]	40.8	4.2
MS + propane[c]	43.4	3.3
MS + *n*-butane[c]	57.7	4.4

[a] Acetylene was present at 0.5% gas phase (vol/vol).
[b] MS, mineral salts medium.
[c] Present at 10% gas phase (vol/vol).

It is important to note that we have not yet determined whether growth of the *Graphium* sp. on *n*-alkanes involves the induction of unique enzymes responsible for the complete oxidation of hydrocarbons or whether these enzymes are constitutively expressed, even in the absence of exogenous *n*-alkane substrates. However, if the enzymes required for *n*-alkane oxidation are present in PDB-grown mycelia, the lack of an effect of acetylene and other unsaturated gaseous hydrocarbons on the growth of the *Graphium* sp. on PDB indicates that the activity of these enzymes does not contribute significantly to the metabolism of this organism under these growth conditions.

Our observation of the inhibitory effect of acetylene and other unsaturated gaseous hydrocarbons on the growth of the *Graphium* sp. may have several implications. First, if, as we suggest, acetylene acts as a mechanism-based inactivator of the enzyme responsible for the initial step in *n*-alkane oxidation, it may be possible to use the covalent binding of carbon from ^{14}C-labeled acetylene as a probe for identifying components of this enzyme. This approach has been very useful for locating the active-site-containing polypeptides of ammonia monooxygenase (9) and hydrogenase (6). Second, our results also indicate that the effects of acetylene on fungal activities should also be considered when this compound is used in field applications as a selective inhibitor of microbial processes.

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TABLE 4. Effect of *n*-alkynes and *n*-alkenes on growth of the *Graphium* sp. on ethane and PDB

Inhibitor[a]	Yield of mycelia on growth medium (mg [dry wt]/100 ml)		
	PDB	MS[b] + ethane[c]	MS alone
None	163.5	20.9	0.5
Propyne	157.4	0.5	0.5
1-Butyne	149.3	0.1	0.8
Ethylene	169.3	0.3	0.3
Propylene	182.3	0.2	1.5

[a] All inhibitors were present at 0.5% gas phase (vol/vol).
[b] MS, mineral salts medium.
[c] Ethane was present at 10% gas phase (vol/vol).

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